PRELIMINARY REPORTS

STABILITY OF TRANEXAMIC ACID AFTER 12-WEEK STORAGE AT TEMPERATURES FROM –20°C TO 50°C

Rodolfo de Guzman, Jr., MT, I. Amy Polykratis, BS, Jill L. Sondeen, PhD, Daniel N. Darlington, PhD, Andrew P. Cap, MD, PhD, Michael A. Dubick, PhD

ABSTRACT

Background. Tranexamic acid (TXA) is an antifibrinolytic agent that reduces blood loss during surgery, decreases mortality in civilian and military trauma populations, was adopted for prehospital use by the British military, and is now issued to U.S. Special Operations Forces for use on the battlefield. **Objective.** This study tested whether storage of TXA ampoules at four temperatures (–20°C, 4°C, 22°C, or 50°C) for 1, 2, 4, and 12 weeks would result in chemical degradation and the loss of activity to block streptokinase-induced fibrinolysis in human plasma. **Methods.** For each temperature and storage duration, normal plasma, plasma plus streptokinase (SK) (50 units/mL), and plasma + SK + TXA (0.2 μg/mL, $n = 4$) were tested for D-dimer (DD), for fibrin degradation products (FDP), by thromboelastography (to measure the units/mL of SK needed to get 100% fibrinolysis at 60 minutes [LY60]), and by high-performance liquid chromatography (HPLC). The results were similar for all temperatures and storage durations, and were therefore combined. **Results.** Streptokinase led to a rise in LY60, DD, and FDP that was significantly (p < 0.05) attenuated with TXA. The results in the three test conditions were LY60: 0.00% ± 0.00%, 70.52% ± 4.7%, 0.02% ± 0.01%; DD: 0.23 ± 0.1, 205.05 ± 101.59, 0.31 ± 0.01 mg/L; and FDP: <10, >40, and <10 μg/mL, respectively. The HPLC results showed no chemical breakdown of TXA. All TXA glass ampoules at –20°C were cracked by week 1. **Conclusions.** Except for the finding that TXA ampoules cracked when frozen, this study indicated that the drug remains effective when stored under conditions likely to be encountered in the prehospital environment and outside the manufacturer's recommended temperature range for at least 12 weeks. **Key words:** tranexamic acid; temperature stability; HPLC; thromboelastography; storage

BACKGROUND

Hemorrhage is the leading cause of death from wounds on the battlefield, accounting for over 50% of deaths.[1,2] Hemorrhage is also a major cause of death in civilian trauma.[3] Evaluations of deaths in Operation Iraqi Freedom and Operation Enduring Freedom (OIF/OEF) have identified hemorrhage as the leading cause of potentially survivable deaths.[2] This has led to a renewed effort to investigate blood products and drugs that improve the efficacy of hemostatic resuscitation, with the eventual goal of having this technology available in the prehospital setting since timely intervention is vital in trauma management.

Tranexamic acid (TXA) is a well-known antifibrinolytic agent that has been shown to reduce blood loss during various kinds of elective surgery.[4,5] It inhibits both plasminogen activation and plasmin activity. The CRASH-2 study, which randomized over 20,000 civilian trauma patients to TXA or placebo,[6] showed that TXA treatment resulted in a statistically significant reduction in mortality. The mortality benefit of TXA was also observed in an observational study of U.S. and British experience in treating combat casualties

1. REPORT DATE **01 JUN 2013**	2. REPORT TYPE **N/A**	3. DATES COVERED **-**		
4. TITLE AND SUBTITLE **Stability of Tranexamic Acid after 12-Week Storage at Temperatures from-20° C to 50° C.**		5a. CONTRACT NUMBER		
		5b. GRANT NUMBER		
		5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S) **De Guzman R., Polykratis A., Sondeen J. L., Darlington D. N., Cap A. P., Dubick M. A.,**		5d. PROJECT NUMBER		
		5e. TASK NUMBER		
		5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **United States Army Institute of Surgical Research, JBSA Fort Sam Houston, TX**		8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)		10. SPONSOR/MONITOR'S ACRONYM(S)		
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION/AVAILABILITY STATEMENT **Approved for public release, distribution unlimited**				
13. SUPPLEMENTARY NOTES				
14. ABSTRACT				
15. SUBJECT TERMS				
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT **UU**	18. NUMBER OF PAGES **7**	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**		

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in Afghanistan.[7] Because of this survival benefit and the low risk of toxicity, the U.S. Joint Trauma System added TXA treatment to its Damage Control Resuscitation Clinical Practice Guideline.[8] A subset analysis of the CRASH-2 data set focusing on outcomes as a function of time elapsed between injury and TXA administration suggested that patients receiving TXA three hours or more after injury no longer benefited from the drug and actually faced an increased risk of mortality.[9] Thus, there is renewed interest by military and civilian physicians in using TXA as early as possible after injury, preferably in the prehospital setting, to improve hemostasis and mortality outcomes in severely injured casualties.

Tranexamic acid is now being issued to U.S. Special Operations Forces for use on the battlefield within three hours of injury. However, the manufacturer's package insert states that the ampoules should be stored at room temperature. Since our military forces are deployed in various environments where ambient temperatures can vary from below 0°C in mountainous areas to above 40°C in desert areas for prolonged periods, there is concern whether the deployed TXA may degrade or become ineffective during prolonged exposure to extreme temperatures above or below the standard manufacturer's recommended storage at 22°C–25°C room temperature. This potential exposure to storage temperatures outside of recommended storage temperatures is also a concern in the civilian prehospital environment where temperatures below –20°C to above 40°C are encountered.[10]

This laboratory study investigated whether storage of the TXA ampoules at four different temperatures (–20°C [freezer], 4°C [refrigerator], 22°C [room temperature], or 50°C [oven]) for five different periods (0 days, 1 week, 2 weeks, 4 weeks, and 12 weeks) would result in the degradation of the chemical structure of TXA and the loss of activity to block streptokinase-induced fibrinolysis in human plasma.

METHODS

This study was conducted as a feasibility study and was not intended to meet U.S. Food and Drug Administration criteria for shelf-life determination of new drugs and products.[11] However, the study was compatible with the Department of Defense 4140.27-M, 2003 *Shelf Life Management Manual*.[12]

To remove the inherent differences among donors in their response to clotting assays as a possible source of variability in this study, a sufficient amount of pooled fresh frozen human citrated platelet-poor plasma (PPP) was obtained from a commercial source (Innovative Research, Sarasota, FL) so that each of the tests described below could be run on the plasma from the same source throughout the study period. The plasma was thawed, pooled, and aliquoted into 50-mL conical tubes and frozen at –80°C until needed. A volume of approximately 30 mL of this plasma was used to evaluate each storage temperature per time point of TXA vial storage: 5 mL per sample with 3 mL used for thromboelastography (TEG), and 2 mL used for measurement of D-dimer (DD) and fibrin degradation products (FDP). The plasma was assayed (BCS, Dade Behring, Deerfield, IL) for prothrombin time, activated partial thromboplastin time, fibrinogen, DD, protein C, von Willebrand's factor, antithrombin III, and factors II, V, VII, VIII, IX, X, XI, XII, and XIII at 1, 2, 4, and 12 weeks, which demonstrated that it remained comparable with data from day 0 (results not shown).

Four ampoules of TXA (Cyklokapron for injection, Pfizer, New York, NY) were stored at each of four temperatures: 1) –20°C (Isotemp Storage Freezer, Model 425F, Fisher Scientific, Hampton, NH), 2) 4°C (Jewett Blood Refrigerator, Model JBB404A, Thermo-Scientific, Waltham, MA), 3) 22°C (Helmer Incubator, Model PC100H, Noblesville, IN), and 4) 50°C (Isotemp Oven, Model 516G, Fisher Scientific), for each of four time points: 1) 1 week, 2) 2 weeks, 3) 4 weeks, and 4) 12 weeks. One set of four ampoules was measured at baseline on day 0 at room temperature.

A preliminary dose–response assay was performed and determined that 50 units/mL of streptokinase (SK) Sigma-Aldrich, St. Louis, MO) was needed to get 100% fibrinolysis at 60 minutes (LY60) as measured by TEG. The amount of TXA (final concentration = 0.2 mg/mL) added to each TEG cup was calculated to be the concentration of TXA that would be present in the plasma of a 70-kg patient with an estimated 70 mL/kg blood volume after receiving the standard 1-g dose intravenously, assuming 100% stayed in the circulation.

On each test day, an aliquot of the human plasma samples was thawed and made up in a volume sufficient to perform the four planned tests: 1) plasma, 2) plasma + SK, 3–6) plasma + SK + TXA ampoules 1–4.

The following four tests were performed: 1) TEG (Haemoscope 5000, Haemonetics Corp., Braintree, MA) measured at 37°C in triplicate. A 1-mL aliquot of plasma was pipetted into a kaolin tube (Haemoscope) to initiate coagulation and 340-μL samples were pipetted into TEG cups with 20 μL of calcium chloride (0.2 mmol/L). 2) D-dimer assay (BCS, Dade Behring) was measured. 3) Fibrin degradation products (Pacific Hemostasis, ThermoFisher Scientific, Waltham, MA) were measured with a semiquantitative latex agglutination assay. For the DD and FDP assays, 2 mL of the plasma was placed into tubes containing Batroxobin (Pacific Hemostasis, ThermoFisher Scientific) and allowed to clot for one hour at 37°C; the sample was centrifuged and the supernatant assayed immediately after separation. 4) High-performance liquid chromatography (HPLC) (Beckman Coulter, Brea, CA) was used to detect evidence for the loss of chemical structure integrity of the product from each of the TXA ampoules as a result of temperature and storage time. A technique validated in our laboratory determined TXA

structural integrity through measures of retention time and peak area. 100 μL of the TXA was combined with 400 μL of 20 mM ammonium bicarbonate, pH 7.5, and 500 μL of methyl cyanide (MeCN). 50 μL (5 mg) of this mixture was injected onto a Luna Amine Column (Phenomenex, Torrance, CA) using 65% MeCN/35% 20 mM ammonium bicarbonate, pH 7.5 for a 15-minute isocratic run. The peak area was measured by ultraviolet absorption at $\lambda = 225$ nm after separation on HPLC.

The TEG, DD, and FDP tested the functional capability of TXA, whereas the HPLC tested the chemical stability of the TXA. TEG analysis gives a functional indication of the efficacy of TXA to inhibit SK-induced fibrinolysis based on actual clot strength. However, TEG measures the change in the viscoelasticity of the clot and cannot differentiate between inhibition of viscoelasticity of the fibrin mesh versus an actual chemical degradation due to SK. We consider this preservation of clot strength to be the most clinically relevant parameter with respect to the ability to prevent bleeding. The DD and FDP tests are specific for the SK-induced fibrin degradation and definitively demonstrated the mechanism by which TXA specifically inhibited the SK-induced breakdown of the fibrin molecule. The HPLC measures the actual chemical stability of the TXA molecule and we specifically looked for a change in peak height and area, and whether breakdown peaks formed. If breakdown was occurring, we expected that the peak height and area would be reduced at the typical retention time of TXA and that breakdown peaks at a different retention times would appear in a reciprocal manner: The additional peaks would appear and the TXA peak would decrease concomitantly.

In order to determine whether the tests used in this study are sensitive to detect a reduced activity of TXA,

we also performed a dose–response curve of the TXA ($n = 4$ ampoules) in PPP to inhibit the 50-U/mL SK dose, using a 1:1, 1:10, 1:100, and 1:1,000 serial dilution of TXA (0.2 mg/mL). The TXA was diluted with saline. For the normal PPP sample, the same volume of saline was added so that an equal dilution of the plasma occurred.

Data analysis was performed using three-factor analysis of variance with post hoc Holm-Sidak tests (SigmaPlot, San Jose, CA). Data are expressed as mean $\pm$ standard error of the means. A $p < 0.05$ from baseline was considered statistically significant.

RESULTS

Thromboelastography

The TEG tracings for each of the tests are shown in Figure 1, and this serves to depict the experimental design. The TXA tracings at each temperature and storage time were superimposable, but were different from the PPP-alone tracing and the PPP+SK tracing. In addition, for each test day and temperature combination, a TEG analysis of the plasma with and without streptokinase was performed to ensure that the streptokinase induced fibrinolysis by 60 minutes, but these results were not included in the statistical analysis. The small changes in the amplitude of the tracings are within the expected variability of this technique. The variability of the SK tracing is also expected because SK causes simultaneous inside-out and outside-in degradation of the clot, and so the initial conformation of the fibrin mesh affects the early pattern of degradation.[13,14] In addition, SK activates not only fibrin-bound plasminogen, but also circulating plasminogen, which can deplete the substrate for fibrinolysis in a variable fashion from sample to
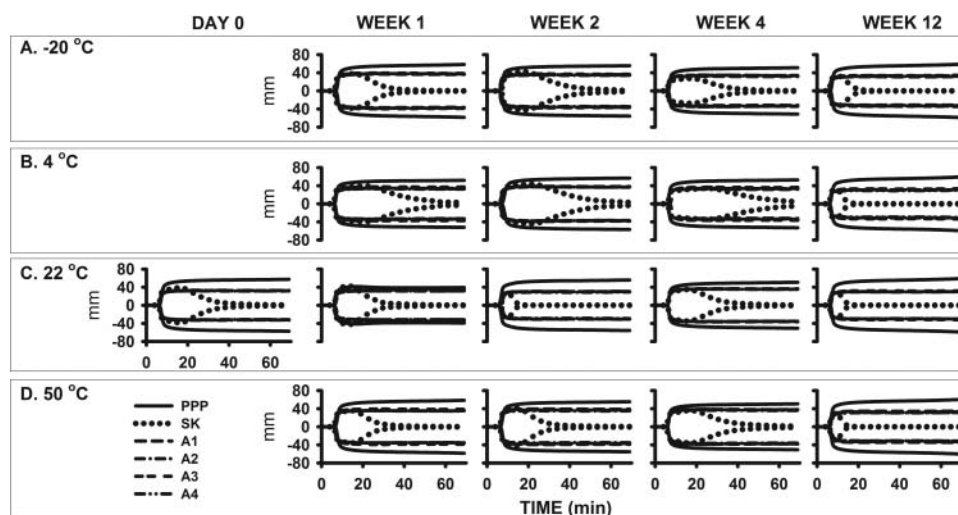

FIGURE 1. Tracings from each thromboelastography run are shown for the four temperatures (**A–D**) at each of the five time periods. A1–A4 = ampoules 1 through 4 of tranexamic acid (0.2 mg/mL) + SK + PPP. A total of 68 ampoules of TXA were used, $n = 4$ for each time and temperature combination. PPP = platelet-poor plasma; SK = streptokinase 50 U/mL.
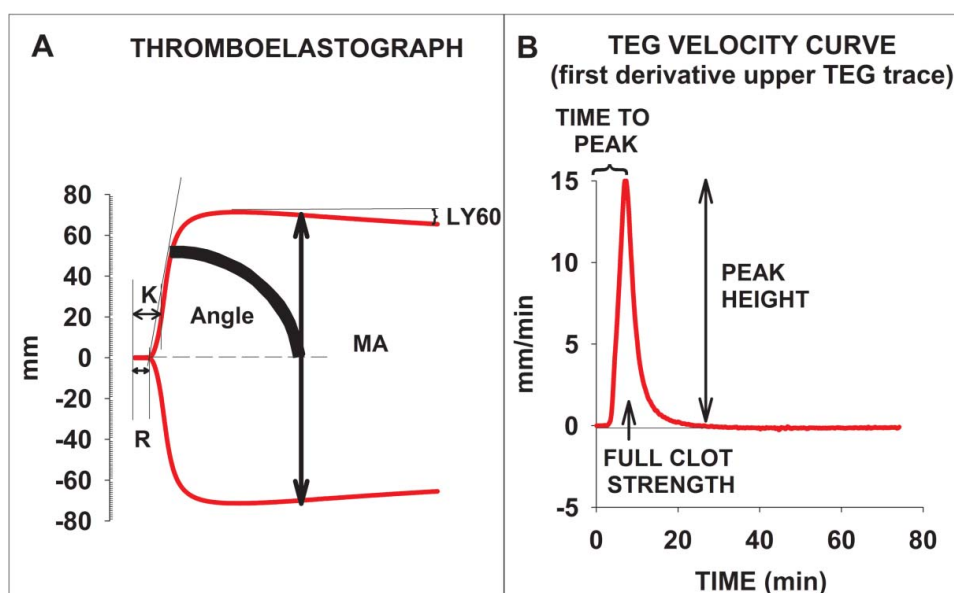
FIGURE 2. Description of the parameters derived from the thromboelastography (TEG) tracing (**A**) and the first derivative of the upper curve (**B**). LY60 = lysis at 60 minutes of thromboelastography.

sample.[15] The dose of SK we used induced 95%–100% lysis by one hour in all tracings. The clinician usually assesses the shape of the TEG curve, so the important thing to notice is that there is 100% inhibition of the SK-induced fibrinolysis in each and every curve.

The TEG parameters that are derived from the TEG tracings are depicted in Figure 2. The TEG parameter LY60 (bottom panel, Fig. 3) was used to evaluate the functional capacity of the TXA to inhibit streptokinase-induced fibrinolysis. Regardless of storage temperature or length of storage, the TXA completely inhibited the streptokinase-induced fibrinolysis. There were no statistically significant differences among the times or temperatures of storage, so the LY60 values were pooled. The LY60 of PPP = 0.00% ± 0.0%; PPP+SK = 70.52% ± 4.7%; and PPP+SK+TXA = 0.02% ± 0.01%.

D-Dimer and Fibrin Degradation Products

There was no significant effect of the storage temperature or duration on the ability of TXA to completely inhibit the streptokinase-induced increase in DD and FDP (Fig. 4), so the values were pooled as before. The DD level of PPP was 0.23 ± 0.1 mg/L and increased to 205.05 ± 101.59 mg/L in the PPP+SK groups. The addition of TXA (PPP+SK+TXA) reduced DD to 0.31 ± 0.01 mg/L. Similarly, the semiquantitative level for FDP in PPP was <10 μg/mL; PPP+SK >40 μg/mL; and PPP+SK+TXA <10 μg/mL.

High-Performance Liquid Chromatography

There appeared to be no physical degradation of the TXA in our evaluation of storage temperatures and
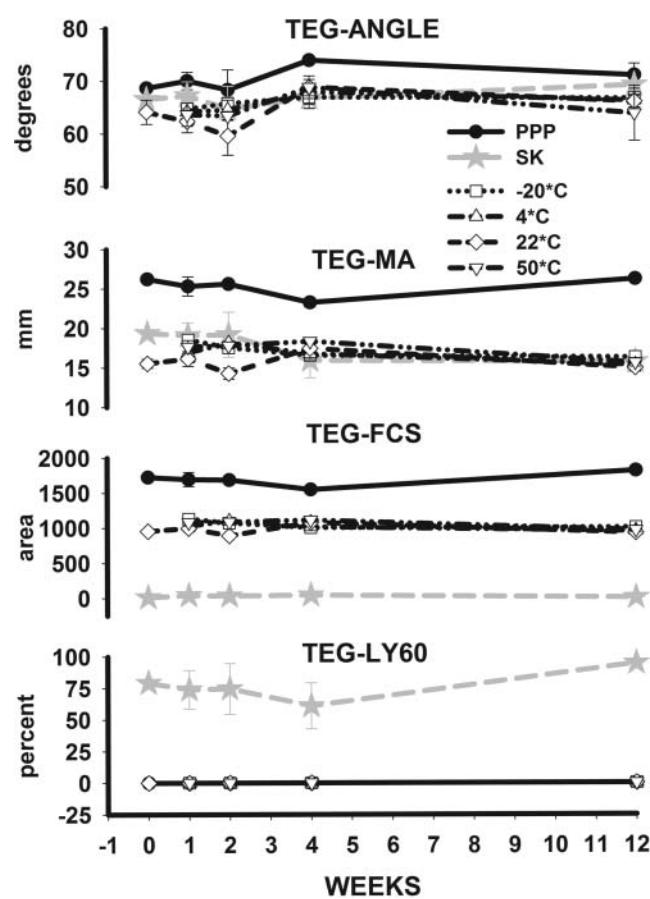

FIGURE 3. Thromboelastography (TEG) parameters. The platelet-poor plasma (PPP) and streptokinase (SK) lines are for reference only. The lines for each temperature are means of four ampoules of tranexamic acid at each point. There was no significant effect of storage temperature or time on any of the TEG parameters. TEG-LY60 = lysis at 60 minutes of TEG.
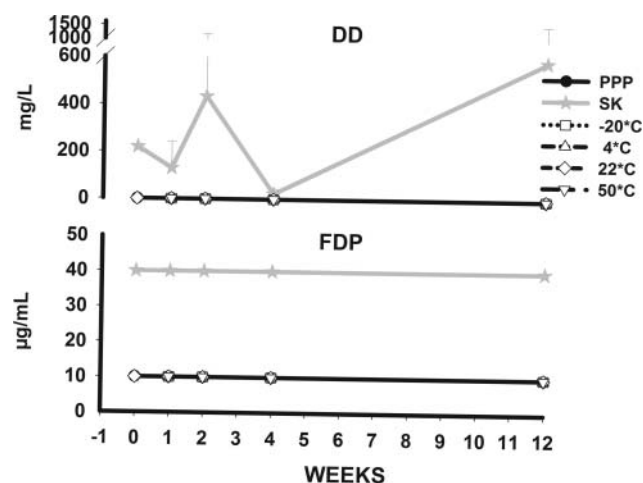
Figure 4. Effect of storage temperature or duration on the ability of tranexamic acid (TXA) to inhibit the formation of D-dimer (DD) and fibrin degradation products (FDP). The platelet-poor plasma (PPP) and streptokinase (SK) lines are for reference only. There was no significant effect of storage temperature or duration on the ability of TXA to inhibit the formation of the fibrinolytic products DD and FDP in streptokinase-treated PPP.
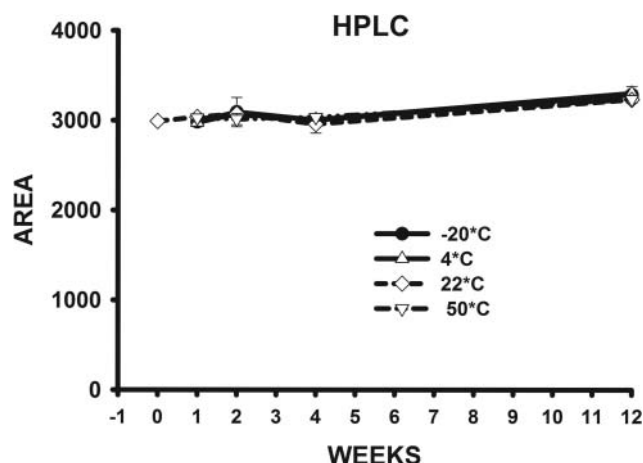

Figure 5. Area under the curve. There was no significant degradation in the area under the peak of the tranexamic acid high-performance liquid chromatography (HPLC) chromatograms with storage time at any temperature.

Figure 6. *Top panel*: representative chromatogram showing an initial elution peak and a main tranexamic acid (TXA) peak. *Middle panel*: enlargement of a typical baseline of a TXA chromatogram. *Bottom panel*: additional noise appeared in all four of the 12-week TXA ampoules stored at –20°C.

duration as shown by the finding of a single peak on the HPLC chromatogram and maintenance of the area under the curve (Fig. 5). However, all TXA glass ampoules stored at –20°C were visibly cracked by 1 week, and showed extra peaks in the baseline of the HPLC chromatograph at 12 weeks (see a representative example in Fig. 6).

Tranexamic Acid Dose Response

The TXA dosing responses are shown in Table 1 (the 1:1 dose was omitted since there was no difference in TEG results from the 0.2 mg/mL dose, DD and FDP

were not performed). Significant differences from PPP were observed at the 1:10 dose for the TEG LY60 and DD, and at the 1:100 dose for FDP.

DISCUSSION

Although TXA has been used for nearly 50 years to control bleeding in surgical patients, it has attracted much interest in the trauma community as a result of the CRASH-2 trial[6] and subsequent studies

TABLE 1.   Tranexamic Acid Dosing Results

Dose	TEG LY60 (%)	DD (mg/L)	FDP (μg/mL)
PPP	0 ± 0	0.7 ± 0.1	10 ± 0
PPP+SK +0.2 TXA 1	0 ± 0	0.8 ± 0.2	10 ± 0
PPP+SK +0.02 TXA 1:10	$0.3 \pm 0.6^*$	$1.9 \pm 0.3^*$	10 ± 0
PPP+SK +0.002 TXA 1:100	$5 \pm 2^*$	$753 \pm 472^*$	$625 \pm 125^*$
PPP+SK +0.0002 TXA 1:1,000	$60 \pm 12^*$	$2,093 \pm 471^*$	$1,000 \pm 0^*$
PPP+SK (no TXA)	$90 \pm 3^*$	$3,093 \pm 390^*$	$1,000 \pm 0^*$

$^*p < 0.05$ different from PPP ($n = 4$).
DD = D-dimer; FDP = fibrin degradation products; PPP = platelet-poor plasma; SK = streptokinase (50 U/mL); TEG LY60 = lysis at 60 minutes of thromboelastography; TXA = tranexamic acid.

indicating that TXA reduced mortality in trauma without increasing the risk of arterial thrombosis.[16,17] In addition, much research has investigated trauma-induced coagulopathy (TIC),[18] which has been identified in 38% of military trauma patients.[19] Recently, TEG has aided in the identification of hyperfibrinolysis (HF) in trauma patients with TIC.[20–23] Although overt HF may be uncommon in trauma patients,[20] occult HF may be more prevalent.[22] Nevertheless, the presence of HF is associated with higher mortality in trauma.[20,23,24] The encouraging results of the MATTERS study[7] and observations that the benefits of TXA are lost if given more than three hours after injury, has generated much interest in the military for prehospital use of TXA. Consequently, considering the varied climatic zones in which the military operates, and the fact that some units may be on isolated missions for extended periods, there is great interest in the stability of TXA if exposed to environments different from the manufacturer's recommended storage at 22°C–25°C (room temperature) for an extended period.

This study demonstrated that the TXA remained functionally stable when stored for up to 12 weeks at temperatures ranging from –20°C to 50°C as determined by the ability of the TXA to completely inhibit streptokinase-induced fibrinolysis of PPP as determined by TEG, DD, and FDP measurements. Furthermore, TXA remained chemically stable when stored for up to 12 weeks at temperatures from 4°C to 50°C and up to 4 weeks at –20°C as determined by there being only a single peak detected by HPLC and no change in the area under the curve from the baseline time 0 sample. The major finding was that 100% of the TXA glass ampoules cracked when frozen at –20°C. However, the frozen TXA remained functionally intact for the entire 12 weeks in its ability to inhibit fibrinolysis, suggesting that packaging the ampoules in something other than glass ampoules would solve this deficiency.

The finding of a noisy HPLC baseline after 12 weeks of freezing may indicate some chemical degradation, although the peak height was not affected. Since the freezer used in the study was frost-free, it is likely that during the defrost cycles, contamination of cracked ampoules may have occurred despite attempts to prevent this through parafilm wrapping of ampoules after

discovery of cracks at week 1. The wrapped, cracked ampoules were placed in conical tubes when thawed to minimize contamination.

In Figures 1 and 3, the maximum amplitude of the thromboelastographs (TEG-MA) was less in the streptokinase-treated samples, even in the presence of TXA, compared with the MA in PPP alone. This may relate to SK's activating both circulating and fibrin-bound plasminogen and the activated circulating plasmin causes fibrinogenolysis to prevent full clot strength.[25] The amount of TXA used in the current study was 1.3 mM, and Hoffmann and Vijgen[25] found that even as high as 70 mM TXA could not inhibit SK-induced fibrinogenolysis.

As mentioned, this study was not intended to meet the Food and Drug Administration guidelines for temperature stability tests for new drugs and products, i.e., testing for a year under constant temperature and humidity. Instead, it was designed to reflect the range of temperatures that may apply during military deployment. These data indicate that TXA retained its functional and chemical stability over a wide range of temperatures for up to 12 weeks, but that efforts should be made to avoid allowing the product to freeze, as ampoules were observed to crack by 1 week and secondary peaks indicating degradation or contamination were detected by HPLC after 12 weeks.

LIMITATIONS

A possible limitation of this experimental design is that the consistent temperatures may not reflect actual prehospital storage conditions in which rapidly fluctuating temperatures exist. Temperature cycling is known to increase degradation of some chemical compounds, especially proteins, but this was not tested in the design of the current study.

Another limitation may be that the functional tests that we used were not sensitive enough to detect small changes in function, although they are the most appropriate tests available. However, as seen by the results of the TXA dosing study, the functional tests showed small but significant changes for the TEG LY60 and DD at a 1:10 dilution (mimicking a plasma concentration of 0.02 mg/mL) and for the FDP at a 1:100

dilution (0.002 mg/mL). The therapeutic plasma concentration of TXA is 0.005–0.015 mg/mL.[26] According to the package insert,[27] the half-life of TXA is about 2 hours and the volume of distribution is about 10 L. Using the standard formula of plasma concentration = total amount administered/volume of distribution, these functional tests are able to detect an 80%–98% degradation in the amount of TXA of a 1-g dose. That is, if instead of 1,000 mg administered, there were an 80% reduction or only 200 mg, the resulting plasma concentration would be 0.02 mg/mL (i.e., 200 mg/10,000 mL), which is the limit of detection for the most sensitive test, DD. Nevertheless, despite a lack of sensitivity in the assays, the tested estimated concentrations of TXA are still above the therapeutic concentrations, suggesting that the drug would still be effective in preventing clot breakdown. In contrast, the HPLC results were linear with dose (unpublished observations, 2012), and therefore would have detected much smaller reductions in concentration. Taken together, the data from the current study support our conclusion that TXA remained functionally and chemically stable at temperatures from 4°C to 50°C for the 12-week period.

These data suggest that prehospital use of TXA, under appropriate medical supervision, is feasible. Clinical practice guidelines could be modified to allow earlier administration (within three hours after injury) of this product and potentially maximize the mortality benefit associated with its use in trauma.

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